THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 69:413–418, 1977

Vol. 69, No. 4
Printed in U.S.A.

FLUX OF FATTY ACIDS DURING EPIDERMAL DIFFERENTIATION

RUTH K. FREINKEL, M.D., AND THOMAS N. TRACZYK, B.A.

Department of Dermatology, Northwestern University-McGaw Medical Center, Chicago, Illinois, U. S. A.

Fetal rat skin prior to cornification (18 days of gestation) was prelabeled with [^{14}C] linoleic acid in vitro and subsequently allowed to differentiate and cornify in organ culture. During 48 hr in culture total epidermal ^{14}C fatty acids and ^{14}C dienes decreased in phospholipids and increased in triglycerides and sterol esters as granular layers and stratum corneum formed. During the second 24-hr period, there was no net loss in ^{14}C fatty acids from the epidermis, suggesting that the changes in phospholipid and neutral esters represented translocations of fatty acids within the cornifying cells. The findings are consistent with the hypothesis that fatty acids liberated by hydrolysis of phospholipids are salvaged by reesterfication as neutral lipid esters during epidermal cornification.

It is recognized that epidermal keratinization is accompanied by a loss of phospholipids and increases in certain neutral lipid esters such as triglycerides and sterol esters [1–3]. Moreover, it is generally accepted that hydrolysis of phospholipids accompanies the degeneration of intracellular membranes during the terminal stages of cornification [4]. Thus, it has been shown that the proportion of sterols in esterified form is increased as fetal epidermis differentiates and keratinizes and is greater in stratum corneum than in living epidermal cells [3,5]. It has also been shown that there is a progressive increase in the free fatty acid and triglyceride content of successive layers of the cow snout epidermis [2]. Furthermore, an increasing proportion of dienes and polyunsaturated fatty acids in the triglycerides of cornified versus uncornified layers has been demonstrated in cow snout epidermis and human skin [6,7]. These findings have suggested that neutral lipids become esterified with fatty acids derived from hydrolysis of phospholipids during keratinization [5,8].

The present studies were undertaken to demonstrate that the above postulate is correct. Fetal rat epidermis obtained prior to cornification was prelabeled with [^{14}C] linoleic acid and allowed to differentiate in organ culture. Flux of ^{14}C fatty acid from phospholipids to triglycerides and sterol esters was documented as the epidermis keratinized in vitro.

Manuscript received March 4, 1977; accepted for publication April 15, 1977

This work was supported by Grant AM 16441 from the Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland.

Reprint requests to: Dr. R. K. Freinkel, Department of Dermatology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611.

Abbreviations:
MEM: minimum essential medium
TLC: thin-layer chromatography

MATERIALS AND METHODS

Tissue. Whole skin from the dorsum of fetal rats at 18 days of gestation was cut into 2 × 2 mm squares under sterile conditions.

Labeling of tissue lipids. Approximately 250 to 300 squares were incubated in 10 ml of sterile culture medium (see below) to which 10 or 20 μCi [^{14}C]sodium linoleate was added. Medium was prepared by addition of [^{14}C]sodium linoleate to 1.0 ml fetal calf serum at 37°C, and subsequent addition of 9.0 ml of minimum essential medium (MEM) (Gibco) culture medium. pH was adjusted to 7.4 with $NaHCO_3$. [^{14}C]sodium linoleate was prepared from [1-^{14}C]linoleic acid (Amersham-Searle) by gentle saponification under N_2 at 60° with sufficient .001 N NaOH to yield a 20% excess of NaOH. The resulting compound yielded a single radioactive spot in routine and argentation thin-layer chromatography (TLC).

Tissues were incubated at 37°C under 5% CO_2/O_2 in a Dubnoff shaker for 1 hr. At the end of incubation, tissues were collected on a sterile nylon mesh filter, washed once with MEM, and incubated for a further 30 min in 10 ml of MEM with 10% fetal calf serum and 0.1 mg cold sodium linoleate/ml. Tissues were again collected on nylon mesh and quickly processed for organ culture.

Organ culture of ^{14}C labeled skin. Six to eight pieces of previously labeled whole skin were placed on sterile squares of nylon mesh, supported by stainless steel grids in the center well of plastic organ culture dishes (Falcon). Enough culture medium was added to just cover the pieces; in several experiments exactly 1.2 ml of medium was added to allow for measurement of the medium radioactivity. Sterile saline was added to the filter paper rings around the center well to assure adequate humidity. At least 24 dishes were prepared and incubated at 37°C in an incubator under 5% CO_2 in air filtered through sterile water. Medium was changed after 24 hr. Tissues were collected at 24 and 48 hr and processed as below.

Culture media, freshly prepared for each experiment, consisted of MEM supplemented with 29.2 mg glutamine, 1.25 ml 7.5% $NaHCO_3$, 10,000 U penicillin, 10,000 μg streptomycin, 25 μg amphotericin B, and 0.2 U pork insulin per 100 ml. Ten percent fetal calf serum

was added to this medium. The pH of the completed medium under CO_2 gas was 7.6.

Processing of tissue. Samples were collected immediately after the labeling incubation and after 24 and 48 hr in culture. Tissues from 4 culture dishes (28–30 pieces) were processed together yielding 3 pooled samples each at 24 and 48 hr. Control samples obtained after the labeling incubation were also processed in aliquots of 28 to 30 pieces.

Epidermis was separated from dermis by peeling with fine forceps after immersing the tissue in 50°C water for 90 sec. Tissues were rinsed in saline, blotted, weighed, and homogenized in 1.0 ml of water 4°C. Aliquots were reserved for protein determination by the method of Lowry et al [9]. The homogenate was extracted in 20 volumes of chloroform:methanol (2:1 v/v) to obtain epidermal lipids.

Analysis of tissue lipids. Total lipids were obtained from the chloroform:methanol extract by the method of Folch et al [10]. Total lipid extracts were dried under N_2, resuspended in chloroform, and aliquots quantitatively applied to 20 x 20 mm silica gel G plates (Merck; Darmstadt) for TLC. Phospholipids, glycerides, sterols, and free fatty acids were separated by 2-dimensional TLC in benzene:ethylacetate (1:1) followed by hexane:ether:acetic acid (70:30:1). Individual phospholipids were separated by 2-dimensional TLC in chloroform:methanol:H_2O:acetic acid (104:40:6:14) followed by chloroform:methanol:H_2O:40% methylamine (65:35:5:5). Spots were visualized with I_2 vapors and identified by reference to R_f's obtained with authentic compounds. Spots were scraped off and counted in Aquafluor (New England Nuclear) in a liquid scintillation counter. All counts were corrected for quenching by the channels ratio method and expressed as dpm. Recovery of radioactivity in the individual lipid fractions separated by TLC and designated in Table IIA ranged from 92 to 93% of the total lipid extract.

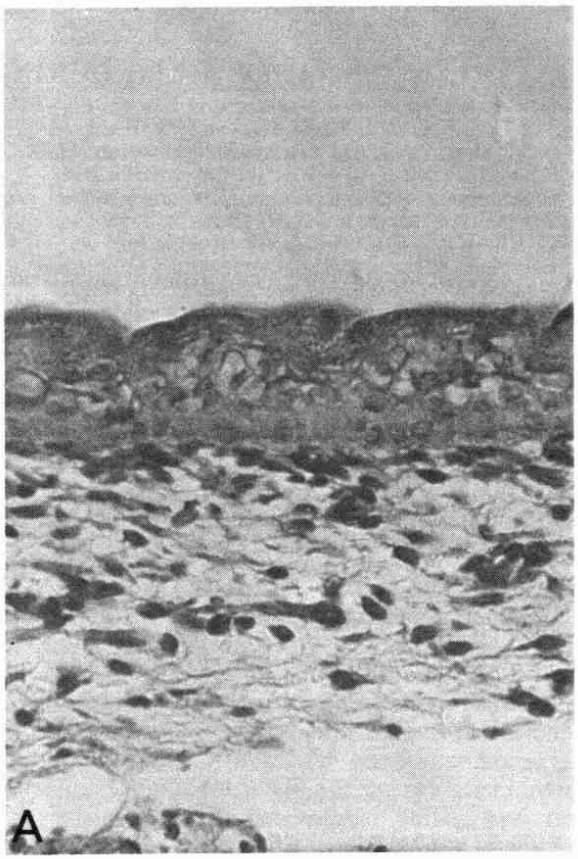
For identification of ^{14}C fatty acids, lipids were separated by TLC as above and spots identified by spraying with rhodamine-6G. Spots were cut out and eluted from silica gel in chloroform:methanol. Eluates were dried and resuspended in chloroform in which the dye is insoluble. Lipids were transmethylated in methyl alcohol with 0.4% H_2SO_4 under N_2 at 60°C for 18 hr. Methyl esters were extracted in hexane and applied to silica gel G plates which had been lightly sprayed with a saturated solution of silver nitrate in methyl alcohol, activated at 110°C for 30 min, and kept dark throughout. Plates were run in hexane:benzene (35:25) and spots identified by spraying parallel authentic markers of stearic, oleic, linoleic, and arachadonic acids with rhodamine 6G. Spots were cut out and counted as above.

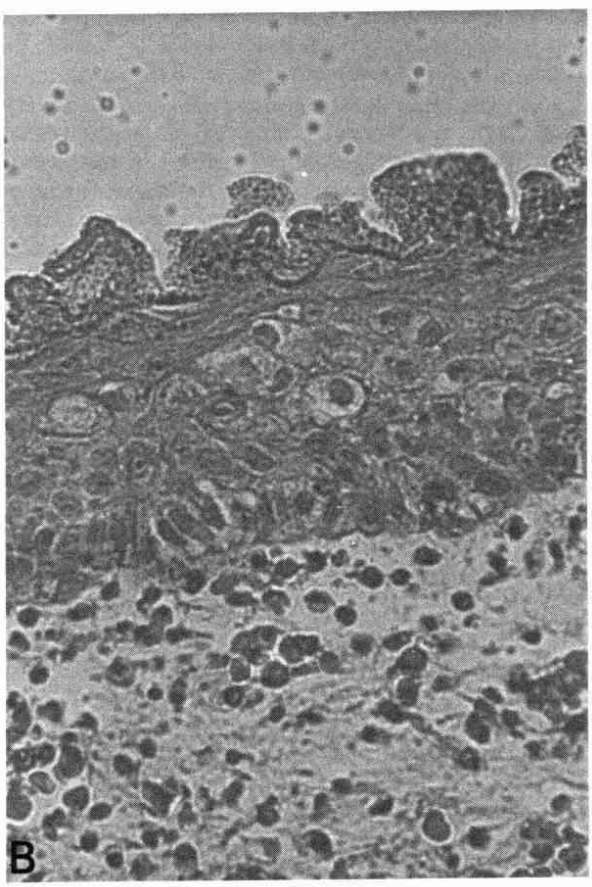
Histology. Representative pieces from each experiment were preserved in buffered formalin and prepared for routine histologic examination with hematoxylin and eosin.

RESULTS

Initial experiments were conducted to characterize the incorporation of [^{14}C] linoleate into epidermal lipids. Some incorporation of ^{14}C into epidermal lipids was observed after 5 min of incubation of whole skin. However, nearly 80% of this represented free fatty acids.

After 60 min of incubation, total ^{14}C lipids increased 4-fold and only 9% represented free fatty acids. Saponification of individual lipids separated by TLC showed that 98 to 100% of the radioactivity





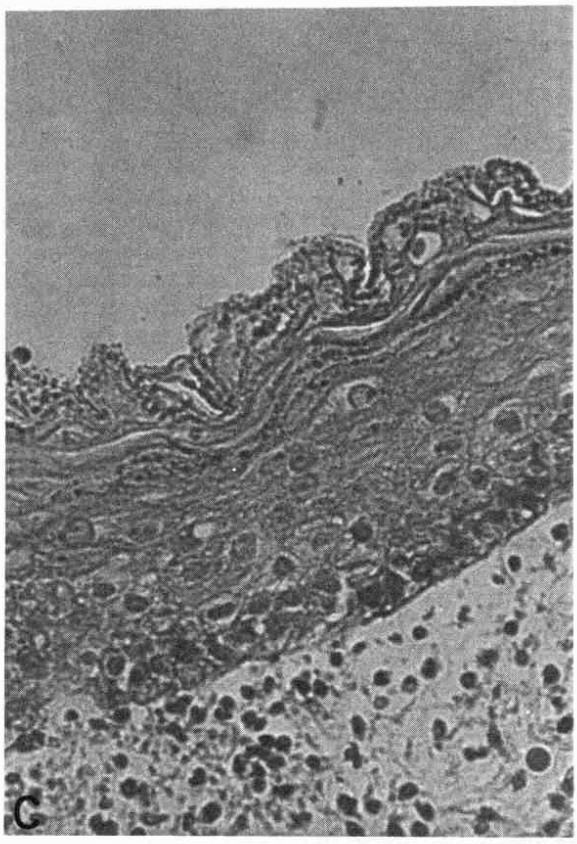


FIG. 1. *a*: Fetal rat skin at 18 days of gestation. *b*: After 24 hr in culture. *c*: After 48 hr in culture (H & E; × 360).

in phospholipids, glycerides, sterol esters, and free fatty acid fractions was present as fatty acid.

Thereafter, experiments were conducted to determine the changes in distribution of lipids labeled with [^{14}C] linoleate after 24 and 48 hr in organ culture. Histologic monitoring revealed that the initially uncornified epidermis (Fig. 1*A*) appeared viable, increased in thickness, and developed both stratum corneum and granular cells after 24 hr in culture (Fig. 1*B*). At 48 hr stratum corneum was thicker, and granular cells were more sparse (Fig. 1*C*). Basal and spinous layers were well preserved, although the dermis showed some degenerative changes.

The distribution of ^{14}C lipid in the entire culture system was explored first. In these experiments culture medium was supplied quantitatively, allowing some pieces to be more fully submerged; this did not affect the progressive changes of developing epidermis. Identical numbers of tissue pieces were used in each culture dish to permit comparable observations based on the total contents of experimental vessels. In a typical experiment whole skin was preincubated with 20 μCi [^{14}C] linoleate in 10 ml of medium. At inception of organ culture the total radioactivity of tissue lipids (epidermis plus dermis) was 10.8×10^5 dpm per vessel with no counts in the culture media (Tab. I). After 24 hr total counts in both tissue and media were 98.6% of the original tissue counts. The loss of labeled tissue lipids could be almost entirely accounted for by appearance of radioactivity in the culture medium. No further significant loss occurred in epidermal lipids after 48 hr; 6.6% of activity was lost from the dermis, and the loss could be almost entirely accounted for by appearance of counts in the medium. These findings suggested that changes in ^{14}C lipid fractions between 24 and 48 hr would represent intraepidermal flux of ^{14}C fatty acids.

The design of the experiments required a reference standard for radioactivity that accurately reflected the amount of epidermis at each time point. It was not technically possible to cut pieces of exactly the same area from the friable and gelatinous 18-day skin to provide comparable areas of epidermis. Wet weight was somewhat variable due not only to differences in size of the explants but also the differences in hydration caused by the absence and presence of stratum corneum. Protein content proved to be the most accurate index of epidermal content. At 0, 24, and 48 hr mean protein content was 105 ± 22, 115 ± 10, and 112 ± 12 μg/epidermal piece, respectively. The differences were not statistically significant although there was a slight increase between 0 and 24 hr while epidermis increased in thickness. This difference was small and would not significantly affect the larger differences in lipid radioactivity (see below).

The distribution of ^{14}C fatty acid in epidermal lipids is presented in Table II*A* and *B* which represents results of three experiments. Values at 0 hr represent mean radioactivity after incubation of tissue in 10 ml of medium containing 10 μCi [^{14}C] linoleate. Total lipid radioactivity per mg protein declined after 24 hr in epidermis but remained nearly constant after a further 24 hr in organ culture. Phospholipids showed the greatest reduction at 24 hr with further decline at 48 hr. Although triglycerides (TG) showed a decline at 24 hr, there was no statistically significant change in any neutral lipids except for an increase in monoglycerides (MG) and sterol esters (SE) ($p < .05$). However, after 48 hr without change in total ^{14}C content, increases over the 24-hr level were noted

TABLE I. *Distribution of ^{14}C label in organ culture system*

Radioactivity (dpm) was determined in total tissue lipids and in the medium before culture (0 time) and after 24 and 48 hr. Medium was changed after 24 hr.

Time (*hr*)	dpm × 10^5/culture dish					
	Tissue			Medium	Total	% Lost
	Epidermis	Dermis	Total			
0	7.10	3.70	10.80	0	10.85	
24	3.55	3.00	6.55	4.10	10.65	1.4
24	3.55	3.00	6.55	0	6.55	
48	3.47	2.80	6.27	0.26	6.53	0.3

TABLE IIA. *Distribution of ^{14}C lipids of epidermis during organ culture*

See text for experimental details. Results of 3 experiments expressed as dpm ± SEM (standard error of the mean) after prelabeling (0 hr) and 24 and 48 hr in culture. Lipid fractions are designated as follows: phospholipids (*PL*); triglycerides (*TG*); diglycerides (*DG*); monoglycerides (*MG*); free fatty acids (*FFA*); sterol esters (*SE*).

Time (*hr*)		dpm/mg protein						
		PL	TG	DG	MG	FFA	SE	Total
0		44,100	11,620	3,590	4,060	6,220	910	70,500
	± SEM	980	790	180	140	244	30	8,500
24		21,530	8,660	3,560	10,490	5,390	1070	50,700
	± SEM	4,400	500	240	1,590	1,200	60	7,060
48		16,850	15,780	3,750	6,910	4,440	1680	49,410
	± SEM	1,400	850	2,250	1,430	1,150	310	6,910

TABLE IIB. *Distribution of ^{14}C lipids of epidermis during organ culture*

Calculations of statistical significance of results presented in Table IIA. Changes (Δ) between 0 to 24 and 24 to 48 hr are based on mean differences of paired experiments. Mean change in dpm ± standard error of the mean difference (SEMD) are shown for each lipid fraction. p values are derived from Student's *t*-test of mean differences in paired experiments.

	dpm/mg protein						Total
	PL	TG	DG	MG	FFA	SE	
			0-24 Hours				
Δ DPM	−22,700	−2960	−30	+6430	−830	+160	−19,800
± SEMD	4,530	1180	330	1380	1590	40	1,530
p	<.05	ns	ns	<.05	ns	<.05	<.002
			24-48 Hours				
Δ DPM	−4,680	+7120	+190	−3580	−950	+610	−1,290
± SEMD	370	720	320	750	265	265	790
p	<.002	<.01	ns	<.05	ns	ns	ns

TABLE III. *Distribution of ^{14}C phospholipids during organ culture*

Results expressed as a % ± SEM of total phospholipid radioactivity in each phospholipid fraction: Phosphatidyl choline (*PC*), phosphatidyl ethanolamine (*PE*); *lyso PC; lyso PE;* and all *others* (phosphatidic acid, sphingomyelin, phosphatidyl inositol).

Time (*hr*)	% dpm ± SEM				
	PC	PE	Lyso PC	Lyso PE	Others
0	64.4	19.8	0.26	4.8	12.0
	±4.2	±2.7	±.03	±.9	±4.0
24	47.5	17.5	1.2	13.9	20.3
	±3.8	±1.8	±.27	±2.8	±5.6
48	48.9	18.4	1.9	9.0	21.7
	±3.5	±2.4	±.58	±4.1	±6.7

in triglycerides ($p < .05$) and sterol esters. Phospholipids and monoglycerides both declined while free fatty acids and diglycerides were not significantly changed. These results suggested a net shift in the fatty acids from phospholipids and monoglycerides to triglycerides and possibly sterol esters.

Changes in individual phospholipids were also investigated (Tab. III). TLC of phospholipids showed that initially phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were most heavily labelled with only 16% of counts in phosphatidyl inositol, sphingomeylin, phosphatidic acid, and in the immediate catabolic products: lyso PC and lyso PE. After 24 hr there was selective loss in phosphatidylcholine and phosphatidylethanolamine with relatively little change in absolute quantities of the other phospholipids which are reflected in increasing proportions of these moieties. Proportions of all phospholipids remained relatively constant at 48 hr, suggesting that phospholipids were equally degraded as keratinization progressed in a stabilized culture system.

Examination of specific fatty acids of various lipid fractions was undertaken. Despite the fact that initially the [^{14}C] linoleate substrate was purely diunsaturated fatty acid, argentation chromatography of the tissue fatty acids after labeling revealed presence of polyunsaturated, monounsaturated and saturated fatty acids in all lipid fractions (Tab. IV). Distribution of the four classes of fatty acids was similar in all the lipid fractions except for monoglycerides after initial incubation with ^{14}C linoleate.

Dienes (representing the initial substrate) were analyzed in all lipid fractions after 24 and 48 hr in culture in two additional experiments. Results are presented in Table V as dpm/mg tissue protein. In

both experiments there was a net loss of dienes after 24 hr of incubation. The change is similar to the overall pattern of tissue lipids presented in Table II; phospholipid diene content declined, that of monoglycerides increased, and in other fractions remained constant or declined. After 48 hr in culture there was no significant net change in total diene content. However, in both experiments there was a further decline in phospholipid dienes accompanied by a greater absolute increase in the triglyceride dienes. The excess increment was largely accounted for by a decline in monoglyceride dienes. Free fatty acid and diglycerides dienes showed variable changes. Sterol ester dienes were increased in both experiments.

DISCUSSION

The present experiments were designed to trace the fate of labeled fatty acids in phospholipids and neutral lipids during differentiation of epidermal cells. Organ culture is well suited to such an experiment since it provides a closed system in which the unkeratinized fetal epidermis progresses to formation of granular and cornified layers. Linoleic acid was chosen as the tracer fatty acid, because it undergoes limited catabolism and is largely retained in the cell as dienoic or polyenoic fatty acid [11]. Thus, it was anticipated that changes in the [^{14}C] linoleic acid content of various lipid compartments would reflect the flux of fatty acid as the prelabeled cells matured and cornified.

In accordance with these anticipations very little of the initial labeled carbon was lost from the entire system, and after the first 24 hr in culture all of the ^{14}C fatty acid present in epidermis was retained.

Changes during the first 24 hr of culture suggested that there was hydrolysis of phospholipids and to a lesser extent of triglycerides with accumulation of monoglycerides. The absence of significant increments in either diglycerides or free fatty acids during this period may reflect rapid breakdown of glycerides and reesterification of free fatty acids as well as diffusion of catabolic products into the medium. It seems likely that the accumulation of monoglycerides is an artifact of the in vitro system since these are usually present in only small amounts in epidermal lipids. The marked loss of both phospholipids and triglycerides also suggests that the early hours of organ culture represent a period of instability following the injury and shock of explanation. Despite this evidence of metabolic trauma, however, the tissue remained viable, showed growth as revealed by its increased thickness, and proceeded towards orderly differentiation.

Morphologic studies during the second 24 hr in culture suggested that the tissue, although apparently viable, was no longer increasing in thickness and that more existing granular and spinous cells had undergone keratinization. Metabolic events during the second 24 hr thus reflected changes in the existing population of cells as these differentiated and keratinized.

These changes lend support to the hypothesis that fatty acids released by hydrolysis of phospholipids are salvaged by esterification as neutral lipids (triglycerides and sterol esters) [5,8]. The fact that the ^{14}C fatty acid content of triglycerides at 48 hr exceeded that at 0 time supports this

TABLE IV. *Distribution of ^{14}C in fatty acids by degree of saturation after labelling with [^{14}C]sodium linoleate*

See text for analytical details. Results presented as % of total ^{14}C fatty acid content and represent a typical experiment. Lipid fractions are designated as in Tables II and III

Lipid fraction	% Distribution			
	Polyenes	Dienes	Monoenes	Saturates
PC	15	60	8	18
PE	17	68	7	8
TG	15	74	4	8
DG	20	60	5	15
MG	19	50	17	14
FFA	18	63	7	12
SE	15	85		

TABLE V. *Distribution of ^{14}C diene fatty acid during organ culture*

See text for details. Results are presented for 2 experiments and expressed as dpm/mg protein. Designation of lipid fractions is that for Table II. △ = net change between 24 and 48 hr.

Time (*hr*)	PL	TG	DG	MG	FFA	SE	Total
			Experiment 1				
0	25,966	9766	2226	1962	3792	833	44,545
24	14,475	5793	1075	6197	3121	850	31,511
48	11,544	11,398	1268	3002	3166	1652	32,030
△ (24–48)	−2931	+5605	+193	−3195	+45	+802	+519
			Experiment 2				
0	30,930	7050	2326	2236	4282	809	47,633
24	8154	6621	1055	4941	1546	669	22,986
48	4914	13,276	1009	1925	1023	870	23,017
△ (24–48)	−3240	+6655	−46	−3016	−523	+201	+31

conclusion. It will be noted, moreover, that the increase in triglyceride fatty acid between 24 and 48 hr equaled the losses of both phospholipids and accumulated monoglycerides. This would suggest that the esterification of fatty acids to triglycerides may be a fairly indiscriminate process involving any available free fatty acids regardless of the source.

Although the data concern only movement of fatty acids in the lipid compartments of keratinizing cells, they are in concert with earlier findings that there are net increases in triglycerides and sterol esters during cornification [2,5]. The data do not suggest that all fatty acids released by hydrolysis of phospholipid are reesterified as neutral lipids. Some may serve to supply oxidative requirements of the cells as has been previously suggested [4]. Indeed, it was to minimize such dissipation that linoleic acid was selected as the tracer fatty acid since this essential fatty acid is not extensively utilized for oxidative processes in other tissues.

The data thus support the view that, in the carefully orchestrated death of the epidermal cell, degeneration of lipid components of cellular membranes (i.e., phospholipids) generates a pool of free fatty acids which are salvaged and sequestered as nonpolar hydrophobic neutral lipid esters. The process is reminiscent of other degenerative processes such as demyelinization and fatty degeneration in a variety of tissues [12,13]. But for epidermis there are some special features which suggest that these changes represent an integral component of keratinization, i.e., production of the dead epidermal cell which from a functional standpoint may be the most important cell in the epidermis. In the first instance there may be no way for the liberated sterols and fatty acids to be released from the cell since the cornified cell retains structural integrity with a vastly thickened cell membrane; sequestration of these polar substances as hydrophobic lipids may therefore serve to preserve the integrity of the cell. Secondly, studies of the diffusion of water and solutes through stratum corneum have suggested that nonpolar lipids play a major functional role in the permeability barrier [14]. Thus, the formation of such hydrophobic lipids as sterol esters and triglycerides from the lipids of degenerating intracellular membranes may serve to illustrate a unique functional adaptation of a more catholic phenomenon associated with cell death.

REFERENCES

1. Yardley HJ: Sterols and keratinization. Br J Dermatol 81 [suppl 2]:29–38, 1969
2. Long VJW: Lipid composition at different depths of cow snout epidermis. Biochem J 114:72–73, 1969
3. Reinertson RJ, Wheatley VR: Studies on the chemical composition of human epidermal lipids. J Invest Dermatol 32:49–58, 1959
4. Long VJW, Yardley HJ: Phospholipids in cultured guinea pig skin. J Invest Dermatol 54:174–177, 1970
5. Freinkel RK, Fiedler-Weiss V: Esterification of sterols during differentiation and cornification of developing rat epidermis. J Invest Dermatol 62:458–462, 1974
6. Long VJW: Changes in the fatty acid composition of the phospholipids, triglycerides, and free fatty acids with depth in the cow snout epidermis. Br J Dermatol 87:227–234, 1972
7. Ansari MNA, Nicholaides N, Hui HC: Fatty acid composition of the living layer and stratum corneum lipids of human sole skin epidermis. Lipids 5:838–845, 1970
8. Freinkel RK: Lipid metabolism during epidermal differentiation, Early Diabetes in Early Life. Edited by R Camerini, H Cole. New York, Academic, 1975, pp 15–20
9. Lowry OH, Rosenbrough WJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951
10. Folch J, Lees M, Sloane Stanley GH: A simple method for isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–505, 1957
11. Aaes-Jorgensen E: Essential fatty acids. Physiol Rev 41:1–51, 1961
12. Dixon KC: Fatty deposition, a disorder of cells. Q J Exp Physiol 43:139–159, 1958
13. Johnson AC, McNabb AR, Rossiter RJ: Chemical studies of peripheral nerve during Wallerian degeneration. Biochem J 45:500–511, 1949
14. Scheuplein RJ, Blank JH: Permeability of the skin. Physiol Rev 51:702–747, 1971